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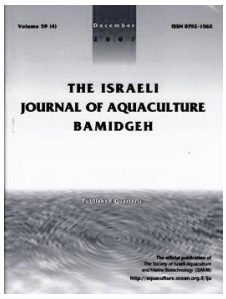

Cinnamaldehyde Altered Cellular Immune Responses of Tongue Sole *(Cynoglossus semilaevis)* In Vitro

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Keywords: cinnamaldehyde; *Cynoglossus semilaevis*; bactericidal activity; leukocytes proliferation; phagocytic activity; respiratory burst

Abstract

Cinnamaldehyde, a liposoluble extract from cinnamon, is a natural compound with immunity enhancement efficacy on terrestrial animals. However, its immunoregulation effects on aquatic animals has rarely been investigated due to its poor water solubility and easy oxidability. Thus, cinnamaldehyde micro emulsion (CME) was prepared to overcome these limitations. Phagocytic, respiratory burst, bactericidal, and proliferative activity of *Cynoglossus semilaevis* leukocytes stimulated by CME were evaluated in vitro. Leukocytes were incubated with 0, 1, 10, 100 and 1000 μg/ml cinnamaldehyde or 100 μg/ml lipopolysaccharide. Results showed that cinnamaldehyde affected leukocytes phagocytic, respiratory burst, bactericidal and proliferative activity significantly. In conclusion, low doses of cinnamaldehyde (1, 10 μg/ml) exhibited significantly high bactericidal activity, while high doses (100, 1000 μg/ml) inhibited cellular immunity of *C. semilaevis*.

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Introduction

Tongue sole, *Cynoglossus semilaevis*, a well-known, high-value marine fish in China (Shang, Ma, & Wang, 2014), is naturally distributed in the Yellow Sea and Bohai Sea of China (Shang, Ma, & Wang, 2014). The rapid growth and large size characteristics of female *C. semilaevis* have increased its farming along coastal region in Hebei, Tianjin, Shandong, Jiangsu, Zhejiang, and Fujian province of China.

However, intensive culture of *C. semilaevis* has resulted in frequent disease outbreaks caused by viruses, bacteria, and parasites. Consequently, fish farmers often suffered mass fish mortality and economic losses (Xiang et al. 2015). On account of the well-known antibiotic resistant pathogen and residual accumulation in tissues caused by antibiotics and chemical disinfectants, enhancing innate fish immunity as a prophylactic measure is of primary concern for fish farmers.

A large amount of immunostimulants, including microbial polysaccharides, plant polysaccharide, chitin and plant extracts, have been reviewed (Cook et al. 2003; Gopalakannan & Arul 2006; and Kirubakaran et al. 2010). Among these promising immunostimulants, plant extracts have been considered appetite stimulators, growth promoters, immunostimulants, and anti-pathogens in fish due to their complicated active principles such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids or essential oils (Reverter et al. 2014). The impact of plant products on innate and adaptive immune system of cultured finfish and shellfish have been reviewed by Harikrishnan et al. (2011), and Awad & Awaad (2017). Among these plant extracts, cinnamaldehyde, which is the main component of cinnamon oil, has been demonstrated to be a promising immunostimulant for terrestrial animals (Bento et al., 2013; Zeng et al, 2015). More importantly, cinnamaldehyde can be mass synthesized artificially with low cost, thus contributing to its practical application. However, there is limited data regarding the immunomodulatory function of cinnamaldehyde on fish. Bathing with cinnamaldehyde was demonstrated to boost expression of innate related genes in zebra fish (Faikoh et al. 2014). Reports of cinnamaldehyde on immune response of *C. semilaevis* have not been found. The objective of the present study was to examine whether cinnamaldehyde affects immune response of *C. semilaevis* leukocytes. Leukocytes phagocytic, respiratory burst, bactericidal, and proliferative activity were tested *in vitro*.

Materials and Methods

Fish.

Eighteen healthy *C. semilaevis* (500 g±50g) were obtained from a recirculating aquaculture system in Tianjin Haifa Aquaculture Co. Ltd. (Binhai New Area, Tianjin, China). Fish were cultured in cement tanks (6.0 m × 6.0 m × 1.5 m). Seawater temperature was 21-22℃, salinity was 20, dissolved oxygen was 8.12-8.92 mg/L, pH was 8.5-8.4 and ammonia nitrogen was below 0.01 mg/L.

Cinnamaldehyde microemulsion (*CME*) *preparation.*

CME was prepared as following: Cinnamaldehyde (Shanghai Richjoint Chemical reagents Co. Ltd., Shanghai, China.) was dissolved in medium-chain triglyceride (Xinxing Pharmacy Limited, Tieling, China) while being stirred at room temperature. Then Tween 80 and 1,2-propanediol (Damao chemical Reagent Factory, Tianjin, China) mixture (mass ratio was 2:1) was added during stirring. Sterile pure water was added drop by drop into the stirred mixture until the mixture was transparent. Micro emulsion (ME) without cinnamaldehyde was prepared following the method above and used as control. Particle size of CME and ME was measured by Zetasizer Nano S90, Malvern.

Preparation of Shewanella algae.

S. algae, isolated from diseased *C. semilaevis*, was used as target pathogen to assay leucocyte bactericidal activity. The pathogen was cultured in beef-extract peptone broth (BPB, Qingdao Hope Biol-Technology Co., Ltd., Qingdao, China) at 28℃ for 24 h, then centrifuged at 2000 g for 10 min, after which the pellets were washed twice in sterile 0.85% sodium chloride and re-suspended in 4℃ BPB. Bacteria suspension was calibrated to 1×10^8 cells/ml before using a bacterial counter chamber and stored at 4℃.

Leukocytes Isolation.

Six fish at each sampling were anesthetized until death with MS-222 (Hangzhou Animal Pharmaceuticals Company, Zhejiang, China). Fish body surface was wiped carefully with clean gauze previously immersed into 75% ethanol (v/v). Blood was drawn from the caudal vein, left to clot in centrifuge tubes overnight at 4℃. After centrifuge, sera were pooled and stored at -85℃ for later use. Kidneys were isolated under aseptic conditions and washed with 0.85% sterile saline to remove blood stains, then put in a 100-mesh sterile cell screen, cut into 5-10 mm^3 pieces with sterilized scissors. The cell screen was placed in a sterile culture dish. A single cell suspension was obtained by gently grinding the tissue with a syringe rod and washed with RPMI-1640 medium which contained 20 IU/ml heparin sodium (Solarbio, Beijing, China), 100 U/ml ampicillin, 0.1 mg/ml streptomycin sulfate and 5% heat-inactivated fetal bovine serum. The resulting cell suspension was moved into a sterile centrifuge tube, centrifuged (600 g, 4℃) for 5 min. The cell precipitation was washed (600 g, 4℃, 5 min) three times with Hank's equilibrium salt solution (HBSS) (Solarbio, Beijing, China). Then, the cells were re-suspended in mRPMI, placed on a 1.020/1.059 Percoll (Sigma, Beijing, China) density gradient, and centrifuged (400 g, 4℃) for 25 min. The isolated leucocytes at the Percoll interface were collected and washed twice with mRPMI. Cell viability was examined by trypan blue (Sigma, Beijing, China) exclusion in a Neubauer hemocytometer. In all samples, cell viability was greater than 95%. The leukocytes were diluted to 2 $\times 10^7$ cells/ml by mRPMI for later use. Leukocytes from six fish were pooled. Three pooled samples were used to assay the respiratory burst, phagocytic, proliferation, and bactericidal activities.

Phagocytic activity.

Phagocytic activity was measured according to the method described by Wang et al. (2011, 2017) with minor modifications. The monolayer phagocytes were incubated for 2 h in mRPMI containing 0, 1, 10, 100, 1000 μg/ml cinnamaldehyde or 100 μg/ml LPS in the humidified 5% CO_2 incubator. After incubation, half of the cells (four wells) from the same sample were fixed with 0.5% (v/v) paraformaldehyde (Tianjin Fengchuan Chemical Reagent Company. Co. Ltd., Tianjin, China) for 60 min and used as the control. Other procedures and calculation followed Wang et al. (2011, 2017).

Respiratory burst.

A nitroblue tetrazolium (NBT) reduction method was used to assay the respiratory burst, following the method of Wang et al. (2017) with minor modifications. The CME was diluted with HBSS to form series of cinnamaldehyde concentration at 0, 1, 10, 100, 1000 μg/ml. One hundred microliters of CME solution at each concentration were added to four wells. Serving as positive control, 100 μl of lipopolysaccahride (LPS, Sigma, Beijing, China) at 100 μg/ml in HBSS were added to another four wells. As blank control, 100 μl of RPMI-1640 medium were added to another four wells.

Bactericidal assay.

Bactericidal assay was measured according to Wang et al. (2017) with some modifications. The final concentration of cinnamaldehyde in wells was 0, 1, 10, 100,1000μg/ml, respectively. Each cinnamaldehyde concentration was repeated in eight wells. Other procedures and calculation followed Wang et al. (2011, 2017).

Leukocyte proliferation.

Leukocyte proliferation was measured according to Wang et al. (2011, 2017) with minor modifications. The final cinnamaldehyde concentration in 96-well plate was 0, 1, 10, 100, 1000 μg/ml, respectively.

Statistical analysis.

Data were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range test with SPSS 18.0 software (SPSS, Chicago, IL, USA). Differences among treatments were considered significant at $P<0.05$. Data in figures were represented as means ± standard deviation (n=3, leukocytes from 6 fish were pooled as one sample).

Results

Cinnamaldehyde micro emulsion (CME) and micro emulsion (ME).

The concentration of cinnamaldehyde in CME was 102.6 mg/ml. The particle size of CME and ME was 93.2 nm and 90.6 nm respectively.

Phagocytic activity.

As presented in Fig. 1, cinnamaldehyde increased the phagocytic activity significantly, among which 1 μg/ml and 10 μg/ml group exhibited the highest activity, followed by 100 μg/ml group. No significant difference was observed between 1000 μg/ml group and control group. As positive control, LPS increased leukocytes phagocytic activity significantly. Compared with LPS (100 μg/ml), the significantly high phagocytic activity in 1, 10, 100 μg/ml cinnamaldehyde indicated that leukocytes were more sensitive to cinnamaldehyde than LPS.

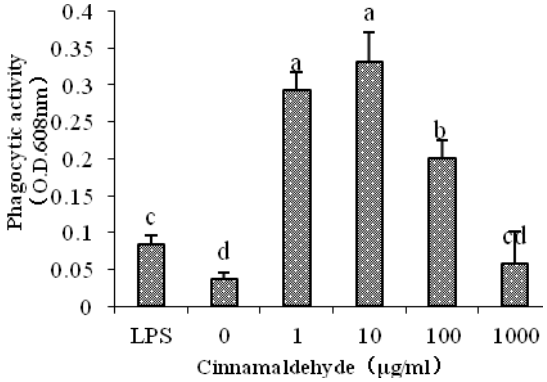

Fig. 1. Phagocytic activity of *C. semilaevis* leukocytes cultured in 100 μg/ml LPS or 0, 1, 10, 100, 1000 μg/ml cinnamaldehyde. Cinnamaldehyde was embedded with micro emulsion. Micro emulsion without cinnamaldehyde served as control. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter are significantly different based on one-way analysis of variance ($P < 0.05$) followed by Duncan's test. LPS, lipopolysaccharide.

Respiratory burst.

Respiratory burst activity is presented in Fig. 2. Cinnamaldehyde increased the respiratory burst activity significantly, among which 10 μg/ml group exhibited the highest activity, followed by 1μg/ml and 100 μg/ml group. As positive control, LPS significantly increased respiratory burst activity of leukocytes, which indicated that the cultured leukocytes were functionally normal in vitro. Cinnamaldehyde at 10 μg/ml exerted statistically equivalent respiratory burst activity with that of 100 μg/ml LPS. This indicated that leukocytes of *C. semilaevis* was more sensitive to cinnamaldehyde than LPS in the aspect of its respiratory burst activity.

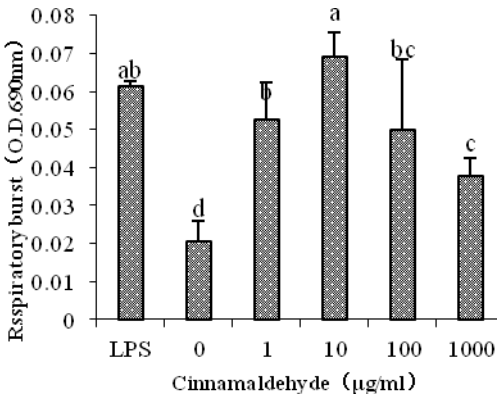

Fig. 2. Respiratory burst activity of *C. semilaevis* leukocytes cultured in 100 μg/ml LPS or 0, 1, 10, 100, 1000 μg/ml cinnamaldehyde. Cinnamaldehyde was embedded with microemulsion. Microemulsion without cinnamaldehyde served as control. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter are significantly different based on one-way analysis of variance ($P < 0.05$) followed by Duncan's test. LPS, lipopolysaccharide.

Bactericidal assay.

The bactericidal activity is presented in Fig. 3. Cinnamaldehyde increased the bactericidal activity significantly at 1 μg/ml. Bactericidal activity in 10 μg/ml and 100 μg/ml group was numerically lower than control, but no statistically significant difference was observed. However, bactericidal activity was reduced significantly by high concentration of cinnamaldehyde (1000 μg/ml).

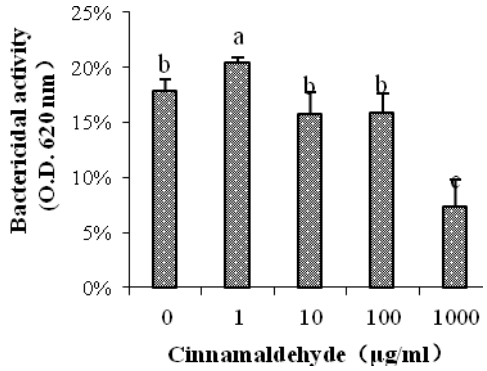

Fig. 3. Bactericidal activity of *C. semilaevis* leukocytes cultured in 0, 1, 10, 100, 1000 μg/ml cinnamaldehyde. Cinnamaldehyde was embedded with microemulsion. Microemulsion without cinnamaldehyde served as control. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter are significantly different based on one-way analysis of variance ($P < 0.05$) followed by Duncan's test. LPS, lipopolysaccharide.

Leukocyte proliferation.

As depicted in Fig. 4, low concentration of cinnamaldehyde (1, 10 μg/ml) led to a significant increase of leukocyte proliferation. However, higher concentration of cinnamaldehyde (100, 1000 μg/ml) reduced leukocyte proliferation. No significant difference was observed between positive control LPS and control group.

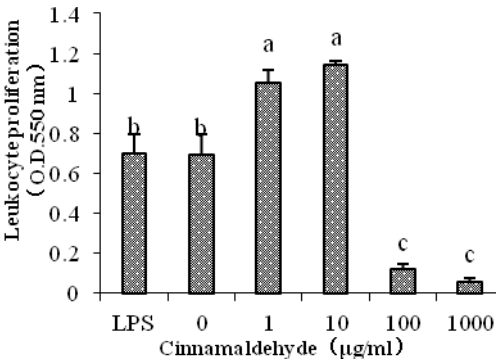

Fig. 4. Leukocyte proliferation of *C. semilaevis* leukocytes cultured in 100 μg/ml LPS or 0, 1, 10, 100, 1000 μg/ml cinnamaldehyde. Cinnamaldehyde was embedded with microemulsion. Microemulsion without cinnamaldehyde served as control. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter are significantly different based on one-way analysis of variance ($P < 0.05$) followed by Duncan's test. LPS, lipopolysaccharide.

Discussion

Plant essential oils are safe and non-toxic (Pavela, 2015). They are generally volatile liquids with aromatic characteristics obtained from aromatic plants by distillation, extraction and other methods (Bento et al. 2013). Studies showed that essential oils possess microbe inhibiting, immune-regulating, and growth promoting activities on aquatic animals (Liu et al. 2018, Cui et al. 2018). However, the complicated component of essential oils is hindered by the illumination of its bioactive mechanism. Cinnamaldehyde, which is an organic compound with the formula C6H5CH=CHCHO, is easily oxidized to cinnamic acid even in blood and hardly dissolved in water. The main purpose of this work was to test the immune response of *C. semilaevis* leukocytes incubated with cinnamaldehyde. In this study, cinnamaldehyde microemulsion was prepared to avoid cinnamaldehyde oxidation and improve its water solubility. The in vitro test demonstrated that the prototype of cinnamaldehyde can dose-dependently affect respiratory leukocyte burst, phagocytic, proliferation and bactericidal activity of *C. semilaevis*. Notably, this is the first study to our knowledge to investigate cinnamaldehyde on cellular immune response of aquatic animals.

Phagocytic, respiratory burst, and bactericidal activity are convincing indicators to assess the immune function of phagocytes (Iwama and Nakanishi, 1996). Our data showed that cinnamaldehyde increased leukocytes phagocytosis, respiratory burst, and bactericidal activity significantly at concentration of 1, 10, 100 μg/ml, 1, 10, 100, 1000 μg/ml, and 1 μg/ml, respectively. Cinnamaldehyde was more effective than LPS in stimulating phagocytic, respiratory burst, and bactericidal activity of *C. semilaevis* leukocytes.

Cinnamaldehyde is a lymphocyte proliferation modulator. Our results indicated that low doses (1, 10 μg/ml) of cinnamaldehyde enhanced leukocytes proliferation significantly. While high doses (100, 1000 μg/ml) of cinnamaldehyde significantly inhibited leukocytes proliferation. Similar results were also found in human peripheral blood mononuclear cells (PBMCs). Low concentration of cinnamaldehyde (0.01-0.05 μg/ml) enhanced viability of PBMCs while higher concentration of cinnamaldehyde (1-10 μg/ml) inhibited viability of PBMCs (Roth-Walter, 2014). In Fig. 4, leukocytes were not significantly responsive to LPS, which is B cell mitogenicity (Sizemore et al., 1984). This inferred that *C. semilaevis* leukocytes could lack analog of B cells in mammals.

In conclusion, this paper provides convincing evidence that 1, 10 μg/ml cinnamaldehyde activated cellular immunity of *C. semilaevis*. These findings contribute to the application of cinnamaldehyde on the healthy farming of *C. semilaevis*. The signal transduction mechanism of cinnamaldehyde on *C. semilaevis* immune cells need further study.

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References

Awad, E., Awaad, A., 2017. Role of medicinal plants on growth performance and immune status in fish. *Fish Shellfish Immunol*. 67:40-54. https://doi.org/10.1016/j.fsi.2017.05.034

Bento, M.H.L., Ouwehand, A.C., Tiihonen, K., Lahtinen, S., Nurminen, P., Saarinen, M.T., Schulze, H., Mygind, T., Fischer, J., 2013. Essential oils and their use in animal feeds for monogastric animals - effects on feed quality, gut microbiota, growth performance and food safety: a review. *Veterinarni Medicina*, 58(9):449-458.

Cook, M.T., Hayball, P.J., Hutchinson, W., Nowak, B.F., Hayball, J.D., 2003. Administration of a commercial immunostimulant preparation, EcoActiva as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (*Pagrus auratus*, Sparidae (Bloch and Schneider)) in winter. *Fish Shellfish Immunol*, 14(4):0-345. doi: 10.1006/fsim.2002.0441

Cui, H., Meng, Y., Zhao, Q., Lv, K., Bian, C., Ma, Y., 2018. A review of current research on use of essential oils in aquaculture. *Fish Sci*, 37(4):564-570. doi: 10.16378/j.cnki.1003-1111.2018.04.021

Faikoh, E.N., Hong, Y.H., Hu, S.Y., 2014. Liposome-encapsulated cinnamaldehyde enhances zebrafish (*Danio rerio*) immunity and survival when challenged with *Vibrio vulnificus* and *Streptococcus agalactiae*. *Fish Shellfish Immunol*, 38: 15-24. dio: 10.1016/j.fsi.2014.02.024

Gopalakannan, A., Arul, V., 2006. Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio*, and control of Aeromonas hydrophila, infection in ponds. *Aquaculture*, 255(1):179-187. https://doi.org/10.1016/j.aquaculture.2006.01.012

Harikrishnan, R., Balasundaram, C., Heo, M.S., 2011. Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture*, 317(1):1-15. http://dx.doi.org/10.1016/j.aquaculture.2011.03.039

Iwama, G., Nakanishi, T., 1996. The fish immune system: organism, pathogen, and environment. In W. S. Hoar, D. J. Randall & A. P. Farrel (Eds.), *Fish Physiology series* (pp. 76-87). California: *Academic Press*.

Kirubakaran, C.J.W., Alexander, C.P., Michael, R.D., 2010. Enhancement of non-specific immune responses and disease resistance on oral administration of *Nyctanthes arbortristis* seed extract in *Oreochromis mossambicus* (Peters). A*quacult Res*, 41(11):1630-1639. doi: 10.1111/j.1365-2109.2010.02516.x

Liu, Y., Liu, W., Guo, X., Chen, Y., Tang, Y., Xiao, H., Li, D., Li, S., 2018. Research progress of plant essential oils in aquaculture. *Feed industry*, 39(8):59-64. doi: 10.13302/j.cnki.fi.2018.08.010

Pavela, R., 2015. Essential oils for the development of eco-friendly mosquito larvicides: a review. *Industrial Crops and Products*, 76: 174-187. https://doi.org/10.1016/j.indcrop.2015.06.050

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., Sasal, P., 2014. Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture*, 433(5):50-61. https://doi.org/10.1016/j.aquaculture.2014.05.048

Roth-Walter, F., Moskovskich, A., Gomez-Casado, C., Diaz-Perales, A., Oida, K., Singer, J., Kinaciyan, T., Fuchs, H., Jensen-Jarolim, E., 2014. Immune suppressive effect of cinnamaldehyde due to inhibition of proliferation and induction of apoptosis in immune cells: implications in cancer. *PLOS one*, 9(10):1-7. doi: 10.1371/journal.pone.0108402

Sizemore, R., Miller, N., Cuchens, M., Lobb, C., Clem, L., 1984. Phylogeny of lymphocyte heterogeneity: the cellular requirements for in vitro mitogenic responses of channel catfish leukocytes. *J Immunol.* 133(6):2920-2924.

Shang, X., Ma, A, Wang, X., 2014. Research progress of Soleoidei in China. *Mar Sci*, 38(12), 142-148.

Wang, Q., Chen, C., Guo, Y., Zhao, H., Sun, J., Ma, S., Xing, K., 2011. Dietary polysaccharide from *Angelica sinensis* enhanced cellular defence responses and disease resistance of grouper *Epinephelus malabaricus*. *Aquaculture International*, 19(5):945-56. https://doi.org/10.1007/s10499-010-9412-x

Wang, Q., Yu, H., Sun, X., Xing, K., Chen, C., Guo, Y., 2017. In vitro activation of *Epinephelus malabaricus* head kidney leukocytes by *Angelica sinensis* polysaccharide. *Isr. J. Aquacult.-Bamidgeh, AquacultureHub,* 69:10.

Xiang, J., Li, X., Chen, Y., Lu, Y., Yu, M., Chen, X., Zhang, W., Zeng, Y., Sun, L., Chen, S., Sha, Z., 2015. Complement factor I from flatfish half- smooth tongue (*Cynoglossus semilaevis*) exhibited anti-microbial activities. *Develop Comp Immunol,* 53: 199-209. https://doi.org/10.1016/j.dci.2015.06.010

Zeng, Z., Zhang, S., Wang, H., Piao, X., 2015. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. *J Anim Sci Biotechnol*, 6(1):7. doi: 10.1186/s40104-015-0004-5